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Sequential accumulation of iron in glial cells during chicken cerebellar development

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ABSTRACT

Iron is an essential, but potentially harmful, metal in the brain. In normal brain, iron has been reported to accumulate mainly in glial cells and occasionally in neurons in some particular nuclei. However, the majority of investigations have targeted the adult brain. Here, we investigated spatiotemporal localization of iron in developing and adult chicken cerebellum using iron histochemistry. Iron reactivity was not detected in the chick cerebellum until embryonic day 12. Iron accumulation was first found in mature myelinating oligodendrocytes located in the inner part of the cerebellar folium at embryonic day 14. From embryonic day 20, iron-positive mature myelinating oligodendrocytes were localized in the white matter and the granular layer. From post-hatching day 2, iron accumulation was observed in Bergmann glia in the Purkinje cell layer as well as in mature myelinating oligodendrocytes. Iron accumulation in microglia was observed in the granular and molecular layers at post-hatching month 12. Our data indicate that during cerebellar development iron is accumulated in a unique sequence according to individual requirements or microenvironmental demands.

Introduction

Iron is the most abundant metal in the normal brain. Within the brain, it is involved in lipid biosynthesis and oxidative metabolism. The myelin sheath of the white matter in the central nervous system (CNS) is comprised of large amounts of lipid. Oligodendrocytes responsible for myelin formation and maintenance in the CNS have high concentrations of iron, essential for lipid biosynthesis (Connor and Menzies, 1990, 1996). Furthermore, oligodendrocytes have high rates of oxidative metabolism. Because heme protein of cytochrome and non-heme proteins (iron sulfur enzymes) engaged in oxidative metabolism contain iron, oligodendrocytes have a higher iron requirement than other brain cells (Connor and Benkovic, 1992; Beard, 2001). Indeed, many studies have shown that iron within the brain is mainly accumulated in oligodendrocytes (Hill and Switzer, 1984; LeVine and Macklin, 1990; Benkovic and Connor, 1993; Erb et al., 1996).

Iron also plays an important role in the synthesis of some neurotransmitters. Glutamate decarboxylase, tyrosine hydroxylase, and tryptophan hydroxylase require iron as cofactors for synthesis of γ-aminobutyric acid (GABA), dopamine, and serotonin, respectively (Youdim et al., 1989; Beard et al., 1993; Li, 1998). Therefore, iron is also accumulated in particular cells of a few nuclei, including the *substantia nigra*, red nucleus, and *globus pallidus* (Benkovic and Connor, 1993; Gerlach et al., 1994).

On the other hand, iron is a potentially harmful element since it may produce free radicals via oxidation. It was established that hydrogen peroxide produced by oxidative mechanisms may form cytotoxic hydroxyl radicals in a non-enzymatic reaction catalyzed by ferrous iron (Fe^{2+}), via the Haber–Weiss reaction (Haber and Weiss, 1934; Gerlach et al., 1994). Therefore, the uptake and storage of iron in the brain is carefully regulated. Alterations of iron uptake and storage have been implicated with neuronal damage in Parkinson's disease, Alzheimer's disease, and Hallervorden–Spatz syndrome (Swaiman et al., 1991; Gerlach et al., 1994; Smith et al., 1997).

Cellular distribution of iron within the brain has been reported in many species including human. Several experiments using rat and rabbit models indicated that iron was accumulated in some astrocytes, microglia, and even in Bergmann glia as well as oligodendrocytes (Benkovic and Connor, 1993; Erb et al., 1996). In a chicken model, iron reactivity was observed in almost all

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oligodendrocytes with occasional accumulation in microglia (Erb et al., 1996). However, the majority of these phylogenetic reports have targeted mature, developed brains. Spatiotemporal distribution of iron in the developing brain remains to be investigated.

The cerebellum originates from the alar plate of rhombencephalon and is a center regulating the movement and position. Birds have distinctly well-differentiated cerebella which are divided by ten primary lobules homologous to the mammalian cerebellum in midsagittal plane (Nickel et al., 1977). The laminar structure and cytoarchitecture of the cerebellar cortex and cellular regulatory mechanism, including inhibitory regulation of GABAergic Purkinje cells, are also homologous to the mammalian cerebellum (Curtis and Stewart, 1986). To investigate cell-specific accumulation of iron within the brain, we characterized spatiotemporal localization of iron in developing and adult chicken cerebellum.

Materials and methods

Experimental animals and tissue preparation

This study was approved by the Institutional Animal Care and Use Committee at Chungbuk National University (Approval No. CBNUA-092-0906-01). Fertilized eggs (Pulmuwon, Korea) were incubated at 38 °C in a humidified atmosphere for the appropriate embryonic days according to the Hamburger and Hamilton stages (Hamburger and Hamilton, 1992). Embryonic cerebella were obtained by cryoanesthetization and rapid decapitation on embryonic days (ED) 12, 13, 14, 17 and 20. Post-hatch cerebella were obtained by deep anesthesia with diethyl ether (Junsei, Japan) and rapid decapitation at post-hatching days (PD) 2, 13 and at post-hatching months (PM) 1 and 12. The meninges of the cerebella were removed in 0.05 M phosphate buffered saline (PBS, pH 7.2). The tissue samples were fixed in 4% paraformaldehyde for 6 h and cryoprotected by 30% sucrose infiltration. Then, the tissues were embedded in freezing compound (Leica Microsystems, Nussloch, Germany) and frozen rapidly. The tissue blocks were cut into 14–16 μm sagittal sections using a cryostat (CM3050S, Leica Microsystems, Germany), and the sections were placed on gelatin-coated slides.

Iron histochemistry

Iron histochemistry was performed according to the method published by Williams et al. (2011). Briefly, the tissues on the slides were washed in Milli-Q ultrapure water and incubated with 0.3% hydrogen peroxide in methanol for 20 min. Then, the tissues were washed in PBS and incubated with the iron stain solution (4% potassium ferrocyanide: 2% Triton X-100: 5N HCl = 1: 2: 1) for 2 h. The sections were washed in PBS and reacted with 0.2% 3,3′-diaminobenzidine (DAB) and 0.1% hydrogen peroxide in PBS. The stained sections were dehydrated, cleared, and mounted with mounting medium (Thermo Fisher Scientific, Waltham, MA, USA).

Immunohistochemistry

The sections were preblocked with 10% normal goat serum for 30 min and incubated overnight at 4 °C with rat anti-myelin basic protein (MBP) antibody (1:300, Chemicon International, Temecula, CA, USA) diluted in PBS. Subsequently, an immunohistochemical procedure was performed using the avidin–biotin peroxidase complex method, as previously described (Seo et al., 2001). Briefly, the sections were incubated with biotinylated goat anti-rat IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:400 in PBS for 2 h at room temperature. The sections were washed in PBS and incubated with the avidin–biotin peroxidase complex (ABC Elite kit, Vector Laboratories). The sections were visualized with 0.2% DAB and 0.1% hydrogen peroxide in PBS, dehydrated, and mounted with mounting medium. For negative controls, the primary or secondary antisera were omitted from the staining procedure.

Double staining by iron histochemistry and immunohistochemistry

For classification of iron-positive cells, we used two cell-specific markers; transferrin binding protein (TfBP) for oligodendrocytes (Cho and Lucas, 1995; Seo et al., 2001) and *Ricinus communis* Agglutinin-1 (RCA-1) for microglia (Mannoji et al., 1986). Following iron histochemical staining, sections were preblocked with 10% normal goat serum for 30 min and incubated overnight at 4 °C with rabbit anti-TfBP (1:500) or RCA-1 (1:3000, Vector Laboratories, USA). The sections were washed with PBS and incubated with biotinylated goat anti-rabbit IgG diluted 1:400 in PBS for 2 h at room temperature. The sections were washed in PBS, incubated with the ABC Elite kit and visualized with SG peroxidase substrate kit (Vector Laboratories, USA). Some iron-stained sections were counterstained with hematoxylin. The sections were dehydrated and mounted with mounting medium.

Results

Iron histochemistry was successful in characterizing cellular localization of iron during chicken cerebellar development. At ED12, no iron-reactivity was observed (Fig. 1A), but a small number of MBP-positive cells were first detected in the cerebellum (Fig. 1B). These cells expressed MBP in cell bodies, and processes extended radially (Fig. 1C). Based on their morphology and antigenic phenotype, MBP-positive cells in this stage could be categorized as non-myelinating oligodendrocytes just prior to maturation (de Castro and Bribián, 2005).

At ED14, faint iron-reactivity first appeared in the inner part of the cerebellar folium, which develops into the white matter in the following stage (Fig. 1D). MBP-positive cells were also observed in the same location (Fig. 1E). Unlike the finding from the previous stage, MBP-positive cells had processes parallel to axons (Fig. 1F). This was a feature typical for mature myelinating oligodendrocytes. The findings from this stage demonstrated that the timing of iron accumulation exactly corresponded to that of oligodendrocyte maturation.

At ED20, iron-positive cells were observed in the white matter and the granular layer of cerebellar cortex (Fig. 2A). The distributional range of iron-positive cells coincided with that of MBP-immunoreactivity in the cerebellum of this stage (Fig. 2B). Iron-positive cells had small and oval-shaped cell bodies with typical oligodendrocyte feature (Fig. 2C). To confirm if these iron-positive cells indeed were oligodendrocytes, we performed double staining by iron histochemistry and oligodendrocyte-specific labeling with anti-TfBP antibody in the ED20 cerebellum. These iron-positive cells were TfBP-immunoreactive, indicating that these cells were oligodendrocytes (Fig. 4A). However, iron-reactivity was not observed in molecular and Purkinje cell layers until ED20 (Fig. 2D).

At PD2, faint iron-reactivity appeared in the Bergmann glia of the Purkinje cell layer, as well as in the white matter and granular layer (Fig. 2E). At PM1, iron-reactivity was increased in the Bergmann glia (Fig. 2F). At PM12, the distribution of iron-positive cells had expanded to all cortical layers including the granular, the Purkinje cell and molecular layers (Fig. 3A). However, MBP-immunoreactivity was restricted to within the white matter and the granular layer (Fig. 3B). In the white matter and the granular layer, iron-positive oligodendrocytes with oval-shaped cell